18. Killifish Hatching and Orientation

Experiment MA-161

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ABSTRACT

In the Apollo-Soyuz Test Project Killifish Hatching and Orientation Experiment, the killifish *Fundulus heteroclitus* was used as a model system for the study of embryonic development and vestibular adaptation in orbital flight. The experiment package consisted of two parts: a series of staged embryos in five individual compartments of a polyethylene bag and a series of preconditioned juvenile fish in a similar bag. Embryos at 32, 66, 128, 216, and 336 hours after fertilization at the time of launch were chosen to represent key stages of development; development preflight occurred at a constant temperature of 295 K (22° C). Juvenile fish were reared from hatching for 21 days in specific visual environments.

Experiment packages were mounted on the docking module wall and photographed periodically during the mission to record the swimming activity of the fish and the condition of the eggs. At splashdown, vestibular sensitivity of the juvenile fish and of hatchlings from the eggs was tested in a rotating, striped drum. Subsequently, additional vestibular orientation tests during parabolic-trajectory flight, light-orientation tests, and geotaxis tests were performed. Samples of juveniles and hatchlings/embryos were fixed for microscopic examination at splashdown and at selected times thereafter. Testing for assessment of normalcy of vestibular function is continuing as the fish mature.

Juvenile fish in a null-gravity environment exhibited looping swimming activity similar to that observed during the Skylab 3 mission. Hatchlings from the 336-hour egg stage also were reported to have looped. At splashdown, both juveniles and hatchlings exhibited a typical diving response suggesting relatively normal vestibular function. The juveniles exhibited swimming patterns indicative of abnormal swim bladders. Rotating-drum tests confirmed that no radical changes in vestibular function had occurred; subsequent tests in maturing fish (6 to 8 months) suggest subtle changes in the geotactic response of the 32-hour flight fish and increased sensitization to environmental influences in 32-, 66-, and 128-hour flight fish as compared to controls. Extensive examination by light and electron microscopy of 66-hour through 336-hour groups has revealed no significant differences between flight and control animals in the embryological development of the central nervous system, the peripheral vestibular apparatus, the eye, or the cardiovascular system. The 32-hour group is still being investigated.

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INTRODUCTION

The Apollo-Soyuz Test Project (ASTP) killifish experiment was the second in a series of experiments to evaluate the hazards inherent in the exposure of living organisms to the space environment during critical portions of the life cycle or for extended periods. The points of primary emphasis were the function and development of the vestibular system and calcium deposition, particularly as it relates to otolith development.

The experimental organism was *Fundulus heteroclitus* (Walbaum), a small shallow-water minnow common along the Atlantic coast. This organism has been used in experimental embryology for many years, and its biology and developmental relationships are well known (refs. 18-1 to 18-3). *Fundulus* was used as a test organism at the NASA Lyndon B. Johnson Space Center (JSC) during the lunar quarantine. Subsequently, the methodology for continuous laboratory production of fertile eggs was developed in this laboratory (ref. 18-4).

The Beaufort, North Carolina, strain of *Fundulus* used in these experiments produces an egg having a transparent, tough chorion. Development may thus be observed from the first cleavage to hatching; and, during this time, the egg may be manipulated extensively without undue stress on the developing embryo. The approximately 2-week period of development is convenient for most laboratory experiments, and the resulting hatchling is relatively large (approximately 1 cm long) with much of the length taken up by the head. The vestibular region, the eyes, and the brain are correspondingly large at this stage. Thus, given the limitations of the available flight conditions, the *Fundulus* embryo presents a useful system for preliminary investigations.

EXPERIMENT BACKGROUND AND DESCRIPTION

A *Fundulus* experiment was included on the Skylab 3 mission in an abbreviated and informal demonstration form. The Skylab package consisted of a plastic bag containing 2 juvenile fish in a compartment with 250 ml of synthetic seawater (21 parts per thousand of dissolved solids) and 50 fertilized eggs (4 days past fertilization) in a separate compartment with 50 ml of seawater. Initially, the juveniles exhibited obvious disorientation reactions (swimming rapidly in loops and circles), but over a period of several days in orbit, they gradually adapted to the weightless environment and to dependence on visual cues for orientation (ref. 18-5). In this state of adaptation, the locker door surface to which the plastic aquarium was attached apparently served as "down." Adaptation was not complete, however, and occasional disoriented swimming activity occurred. Nearly all the 50 eggs hatched in space, and because of a delay in hatching of the flight fish, several returned alive to Earth. However, a series of unfortunate events resulted in the death of these animals shortly after return. The space-hatched *Fundulus* fry exhibited no observable tendency toward disoriented swimming activity, and their apparent dependence on visual orientation cues both onboard the Skylab spacecraft and on the recovery ship suggested the possible absence of vestibular input. Preservation of the returned hatchlings was insufficient to prevent deterioration and did not permit definite conclusions regarding the condition of the vestibular system. Typical otoliths, however, were not visible in microscopic sections of the flight fish.

The ASTP killifish experiment was designed to confirm and extend the Skylab 3 observations of vestibular disturbance and embryonic development. Calcium loss has been observed in humans after exposure in space, and otolith development is, in fact, largely concerned with calcium metabolism. Therefore, observations of otolith development are important not only because of the importance of the vestibular system in balance but also because of the possible use of the otolith as a model for space research on calcium metabolism in general.

The Skylab observations also raised several questions regarding mechanisms of vestibular disturbance and adaptation in the *Fundulus* and its usefulness as a model for vestibular research. The primary questions asked in the ASTP experiment were related to the rate of adaptation, which is only sketchily known from the Skylab experiment, and the role of the visual system in orientation and adaptation to null-gravity conditions.

Experimental Design

The experiment consisted of a two-part experimental package (fig. 18-1) that was exposed in orbital flight and of four control treatment packages. Except for the treatment designated "launch control," all packages contained the experimental materials enumerated in table 18-I. The following control packages were used.

1. Transport control (package C-I): This package was transported with the flight package. During the mission, it was exposed to a constant temperature (296 K(23° C)) and to the predicted atmospheric pressure and oxygen (O_2) partial pressure profile of the ASTP spacecraft. Recovery processing and return to JSC paralleled those of the flight treatment package.

2. Laboratory air control (package C-II): Immediately after packing, the fish and egg packages were placed in a 295-K (22° C) incubator under standard atmospheric conditions until recovery. Subsequent handling was similar to that for the flight treatment package.

3. The 48-hour-lag control in ASTP-simulated air (package C-III): Eggs were fertilized and packages prepared on a schedule precisely 48 hours behind the flight schedule. All timing and conditions of preparation of flight packages were duplicated. At launch time, the package was exposed to a simulated launch vibration profile and then placed in an environmental chamber for exposure to simulated spacecraft atmosphere. Atmosphere (O_2 partial pressure) and temperature were programed to duplicate the averaged readings of the spacecraft sensors. Recovery activities paralleled those of the flight treatment package.

4. Launch control: Smaller samples of eggs (10) and fish (2) were prepared in parallel with the flight controls and the 48-hour-lag controls and were transported with these treatment packages. At lift-off, these samples were fixed in cold trialdehyde fixative to provide a record of developmental condition at lift-off.

FIGURE 18-1.—Experimental package consisting of fish packet and egg packet.

TABLE 18-I.—Experimental Packages

(a) Egg package with five compartments

Compartment	*Number of eggs*		*Age of embryos at launch, hr (a)*	*Nominal stage of embryogenesis*
	Launch	*Return*		
1	100	100	32	Mid-to-late gastrulation
2	100	100	66	Statolith precursors appear
3	100	100	128	Vestibular development completed
4	100	100	216	Vestibular system functional
5	50	50	336	Full development; able to hatch

(b) Fish package with five compartments

Compartment	*Number of fish*		*Preconditioning*	*Flight background*
	Launch (b)	*Return*		
1	6	5	Neutral background	White
2	6	5	Vertical bars	Barred
3	6	5	Horizontal bars	Barred
4	6	[c]0	Black overhead	Black
5	6	6	Blinded	White

[a]Development occurred at a constant temperature of 295 K (22° C).
[b]Age of fish at launch was approximately 21 days.
[c]All fish in this compartment were dead.

Experiment Theory

The embryo development portion of the experiment was designed to isolate the effects of exposure to a null-gravity environment in progressively more advanced stages of embryo development. The ages were selected as probable average times for the onset of key stages of development with particular reference to the vestibular system and otolith development. Development time varies somewhat within the population but is essentially complete by 9 to 11 days, and most of the eggs may be stimulated to hatch at the end of 14 days under standard laboratory conditions. Thus, in a majority of the flight treatment eggs, all subsequent embryogenesis was completed in the near-null-gravity conditions of orbital flight. The three later stages, 128, 216, and 336 hours, were theoretically capable of hatching during the mission. One experiment objective was to observe and record swimming activities of the hatchlings from these eggs to confirm the observations of apparent adaptation in the Skylab hatchlings.

Embryos of different ages (table 18-I(a)) were obtained by stripping ova and milt from adult *Fundulus* (ref. 18-4) and combining these to effect fertilization at the given prelaunch times. Fertilized eggs were then incubated in plastic petri dishes containing synthetic seawater (21 parts per thousand of dissolved solids) at a constant temperature of 295 K (22° C). Each petri dish contained a maximum of 50 eggs that were washed daily with filter-sterilized synthetic seawater to ensure removal of metabolic products and to reduce the population of contaminant

microorganisms. Between 30 and 26 hours before launch, all eggs were sorted, given a final wash, and sealed into the compartments of the plastic flight package.

The vestibular adaptation portion of the experiment was designed to extend previous observations of *Fundulus* swimming behavior in a null-gravity environment. The cause of the observed disorientation in the fish is largely unknown, although the vestibular system is presumed to be primarily responsible. The observed adaptation during the Skylab 3 mission was apparently based partly on a switch to dependence on the visual system for orientational cues. The entire process is of more than passing interest because it parallels in many ways the patterns reported for human adjustment to weightlessness, and findings regarding vestibular adaptation in fish could usefully be applied to humans (ref. 18-5). Because of time and space limitations, the experimental scheme was not intended to produce profound conclusions; it was simply an attempt to refine previous measurements of rate of adaptation and to define the nature of the visual stimulus (light direction or background surface) in the apparent habituation of fish to null-gravity conditions. Of equal importance was the observation of readaptation of the partly space-adapted fish to one-g conditions.

The experiment package consisted of several small plastic-film bags (fig. 18-1), each containing a number (table 18-I(b)) of *Fundulus* fry (immature fish 21 days old at time of launch) that had been reared from hatching in tanks presenting different visual environments. The swimming activity of the fish was recorded with a motion picture camera at various times during the flight with the objective of measuring general rate of adaptation as well as orientation patterns and relative rates of adaptation of the visually conditioned fish.

Data Collection

The data yield of the experiment consisted of visual observations from the flight, motion picture recordings of the condition and activity of experimental animals during and after the flight, animals for microscopic examination that were fixed at recovery and at subsequent selected sampling times, and results of behavioral tests taken at recovery and at various times during the maturation of the fish.

The primary objective of the histological sampling was to detect anatomical differences in the flight animals that could be correlated with behavioral differences and to record any observable readjustments that might occur in the developing animals after their return. Samples of juvenile fish were fixed in trialdehyde fixative at splashdown and several weeks later following completion of behavioral testing. To provide a picture of progressive changes, hatchlings and unhatched eggs were sampled at recovery, at approximately daily intervals for the first week after splashdown, and again at 2 weeks, 1 month, and 6 months. Analysis involved, as a first stage, serial sectioning of the fixed animals for light and electron microscopy of the head and body cavity and examination for anomalous development of the vestibular apparatus, the central nervous system, the eye, and the cardiovascular system. Of particular interest was the degree of calcification of cartilage and of the otoliths.

Postflight testing was performed on both fry and hatchlings from the flight and control treatment packages with the objective of detecting and measuring differences in orientation behavior attributable to null-gravity exposure during development. The tests employed were the rotating striped-drum test, the light-orientation test, the geotaxis test, and brief exposure to null-gravity conditions during parabolic-trajectory flight. A final test, to be conducted when the fish are fully mature, will measure and analyze locomotor activity.

The classical fish otolith tests (ref. 18-6) were based on measurements of the fish longitudinal-axis tilt from the horizontal when the direction of water currents or of illumination is varied. At the University of Mainz, an additional otolith test that works especially well with very young and small fish has been developed. The fish tank containing the unrestrained fish is placed inside first a vertical and later a horizontal rotating striped drum. In the vertical drum, the fish visually lock with the stripes and swim around inside the drum, keeping pace with the moving stripe (fig. 18-2(a)). In the horizontal drum, the fish are prevented from

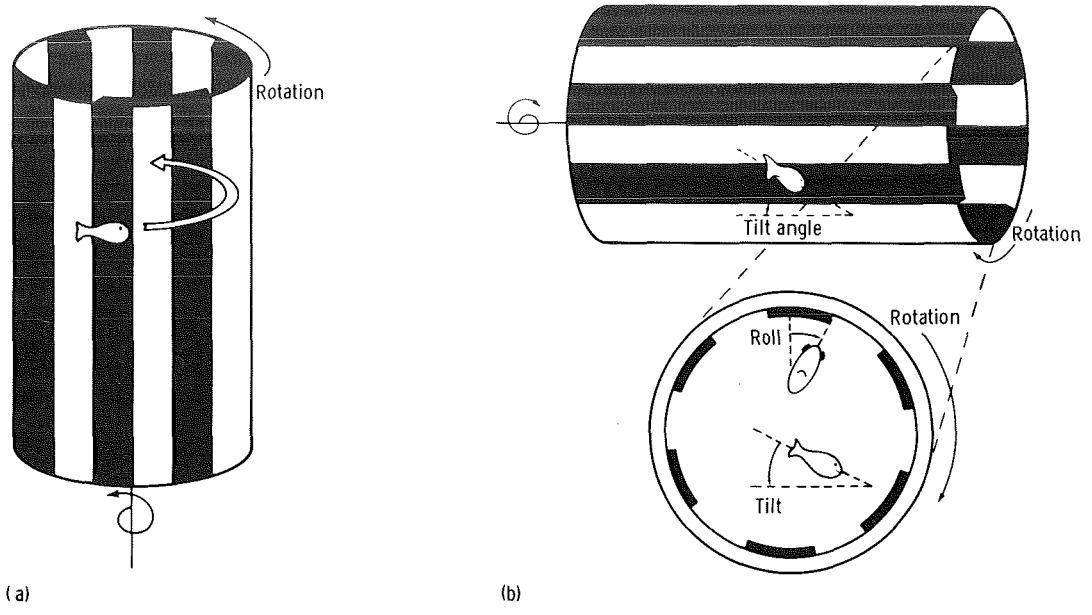


FIGURE 18-2.—Orientation in rotating drum. (a) Vertical position. (b) Horizontal position.

looping solely by action of the otolith system (fig. 18-2(b)). However, if the fish face the stripes, their bodies are tilted in the direction of stripe movement. The fish that are turned 90° away from the stripes roll to a measurable degree in the direction of the drum rotation, but again are prevented from tilting further by the action of their otolith system. Abnormal tilt indicates a lowered sensitivity of the otolith system. The rotating-drum test was employed as the initial vestibular test given to the returned flight fish on the recovery ship. Testing began within 8 hours of splashdown and continued for 5 days. In this test, the fish were inside a sealed tank that was placed inside the drum for varying times of at least 1 minute in both horizontal and vertical drum positions. Tilt angle and movements were recorded by a motion picture camera for later single-frame analysis.

In addition to the rotating striped-drum test, a light-orientation test was given to the juvenile fish to further evaluate their ability to orient in the geogravitational environment after a 9-day exposure to weightlessness. Although it is well known that fish maintain their relative vertical orientation by integrating sensory information from the influences of both gravity and light (ref. 18-6), it was not known whether the exposure only to light cues during a 9-day period would affect this integration when the fish were reintroduced into the normal one-g environment.

In the normal geogravity environment, fish maintain their equilibrium orientation by static and optic stimuli. If these stimuli do not operate in the vertical direction, the fish takes a position between the vertical plane and the incidence of the light (ref. 18-7). For example, if a light is placed in a darkened chamber at a 90° angle relative to the vertical plane, the fish will roll about its body axis toward the light and the dorsal fin will form a 30° to 60° angle relative to the vertical plane. (The exact magnitude of the angle varies with species of fish and other environmental factors such as light intensity and dark adaptation.) Normally, fish live in an environment in which light is overhead and therefore represents a reference for the vertical in addition to the sensing of gravity. The fish rolls toward the light source to accommodate its sensory inputs in the new 90° test environment. If the light intensity is increased, the fish will roll even farther toward the light, up to a point. If gravity is increased (as in a centrifuge), the fish will roll back toward the gravitational vertical. The static vestibular sense is

the more dominant of the two systems, but if it is damaged (as in ablation studies), the fish will orient strictly with respect to the dominant light direction. Therefore, the light-orientation test was an attempt to measure a possible modification of the gravitational receptors due to a 9-day exposure to null-gravity conditions. In this test, each juvenile fish was placed in a clear plastic cylinder and its dorsal fin orientation was measured after a 40-W bulb was placed 14 cm to the right of the fish (90° relative to the vertical plane) for 30 minutes and then to the left for the same period. Dark adaptation was permitted for 1 minute before each test interval, and right and left tests were randomized. Each fish was given one trial (left and right orientations) in any given test day. All fish were tested sequentially until four trials per fish were made over a period of several days.

A geotaxis test developed at JSC was employed for further testing of maturing fish after 6 months. In this test, a fish is gently placed just below the meniscus of the synthetic seawater in a graduated cylinder filled to the 500-ml mark. The amount of time spent in the upper half of the cylinder is recorded over a 2-minute interval as a measure of the geotaxis response. This diving response is very strong in most *Fundulus heteroclitus*; therefore, the test was a measure of whether or not this innate vestibular program was modified by an exposure to null-gravity conditions during embryonic development.

Experiment Execution

Execution of the flight portion of the experiment was, relatively, the simplest part. The prepared flight package was transported to the launch site in a T-38 aircraft between 26 and 23 hours before launch and placed in a storage locker of the command module approximately 9 hours before launch. At approximately 30 hours after launch, the metal and glass cloth flight container was opened and the experimental packages were mounted on the docking module wall for the duration of the mission. Motion pictures were taken of the fish package at this time. No photography or observations were scheduled during the joint phase of the mission because of the heavy demands placed on the crew during that time. Daily observations were recorded in the flight notes, and motion pictures were made on mission day 6 and all subsequent days of the mission. Following photography on mission day 9, the packages were returned to the flight container and stored for reentry.

The flight package was removed from the command module 6 hours after recovery and was photographed to record initial swimming activity. Hatching was induced by agitation on a rotary shaker, and samples of eggs, hatchlings, and juvenile fish were killed and fixed for microscopic examination. Additional samples were fixed at approximately 24-hour intervals for 7 days after recovery, at 2 weeks, and again at 1 month. Upon return of experimental materials to the laboratory 2 days after recovery, selected specimens were injected with tritiated thymidine and killed after a 1-hour exposure.

Testing for vestibular sensitivity by the rotating-drum method was begun as soon as the juvenile fish and hatchlings were removed from the flight packages and was continued for several days after splashdown. At 5 days, selected groups were tested for orientation in parabolic-trajectory flights. Continued testing of the animals remaining after sampling for histology included light-orientation tests during the first several weeks following splashdown and tests of geotactic response at 6 to 8 months. A final series of tests to be conducted when the fish reach a length of approximately 7.5 cm will include analysis of oriented locomotor behavior patterns as a final sensitive assay of alterations in the vestibular system.

Following return of the experimental materials to JSC, hatchlings and juveniles were maintained in small plastic dishes containing aerated synthetic seawater. After several weeks of observation and light-orientation testing, all juveniles were fixed. Initially, the hatchlings produced after each daily shaking were maintained separately, but after the early intensive sampling for histology, the various daily hatches were pooled. After approximately 2 months, hatchlings were transferred to polycarbonate mouse cages and critical groups including the 32-, 66-, and 128-hour

hatchlings of flight and control animals were separated out and maintained under a 16:8 light-dark cycle. During geotaxis testing, all animals were also adapted to a 12:12 light-dark cycle so that measurements could be made on the same subject under the influence of two different photo periods. At 9 months, the members of the critical groups were separated into individual containers and thereafter maintained on a 12:12 light-dark cycle.

There are currently no plans for further sacrifice of animals for microscopy until other aspects of the experiment have been completed. Surviving animals are being raised to maturity; when they are of adequate size, they will be tested for possible alterations in oriented locomotor behavior by employing the techniques and apparatus of Kleerekoper (refs. 18-8 to 18-10). Upon completion of behavioral testing, egg and milt production will be induced (ref. 18-4) and a second generation initiated.

RESULTS

Because of the long-term nature of some observations and the complexity of analyses, this report does not present the final results of the experiment. Status of the experiment is updated, preliminary results and expected trends are reported, and the directions to be taken in completion of the experiment and in planning future sequels to this experiment are suggested.

Vestibular Behavior of Juvenile Fish

In-flight activity.—Three points were examined with respect to orientation in the juvenile fish: the influence of previous environment on initial orientation behavior during the flight, the ability to adapt to weightlessness and the nature and relative importance of the cues employed in adaptation, and the effect of exposure in flight on the ability to orient after return to a one-g environment. Initial observations of orientation were lost because of an improperly seated camera magazine. Subsequent observations on mission days 6 to 9 showed looping activity similar to that observed on the Skylab 3 mission. Single-frame analysis of the flight films indicate that, by day 6, the fish were partially habituated and were largely orienting belly to the wall or back to the camera. Looping appeared to occur only after disturbance of the flight package. The data suggest that sighted controls (compartment 1) and blinded controls (compartment 5) showed a greater tendency to loop than those reared in the presence of specific visual environments. Fish in compartment 4 died of unknown causes before the end of the mission; hence, observations on these should be suspect. Although numerical trends seem to suggest some effect of visual conditioning on adaptation, analysis of the data (table 18-II) indicates no statistically significant difference among treatments.

Recovery.—Upon opening of the flight package 6.5 hours after splashdown, juvenile fish exhibited

TABLE 18-II.—Effects of Visual Preconditioning on Adaptation as Measured by Numbers of Fish Looping Over 4 Days of Observation

Compartment	*Treatment*	*No. of fish looping*	*Total fish*	*Proportion of looping fish*	*Time filmed, sec.*	*Value of F*	
						Calculated (a)	*Tabulated (b)*
1	Normal control	10	20	0.50	41	--	--
2	Vertical bar acclimated	8	20	.40	78	1.39	2.28
3	Horizontal bar acclimated	5	20	.25	91	2.56	2.77
4	Black overhead acclimated	2	14	.14	82	4.12	5.80
5	Blinded	11	17	.65	102	1.11	2.07

[a]Compared to controls.
[b]The significance level is 5 percent.

typical fright-diving responses. After recovery from their initial fright, most fish swam belly down in a nearly normal position. No marked impairment of the vestibular function could be deduced from the spontaneous swimming pattern. Slight differences of behavior compared to the behavior of control fish were observed. During the first hours after space flight, the experimental fish spent more time at the bottom of the bags or at the bottom of the tank in the test drum than did the controls. The specific density of the experimental juvenile fish seemed slightly increased compared to the controls, as was deduced from the faster sinking rate between movements and the steeper angle of attack during periods of horizontal swimming. However, these differences were small compared to the control fish, and further single-frame analysis is required to determine whether this observation is statistically significant. Investigation of the development of the swimming bladders in the juvenile fish that were fixed for histological examination would be interesting and might confirm the hypothesis for this behavior.

Striped-drum test.—The first striped-drum tests of the experimental fish were conducted approximately 6.5 hours after recovery. Tests were performed with fish from compartments 1, 2, and 3; the fish in compartment 4 were dead on recovery. The fish of compartment 5 were blind and therefore could not be optokinetically stimulated. The juvenile fish at this time showed no significant change of vestibular behavior when compared to the behavior of the control fish. Tests of the recovered fish in the vertical drum with horizontal movement of the stripes showed that all tested fish followed the horizontal stripe movement well and swam in continuous circles around the axis of the drum. From this observation, it can be concluded that the optomotoric function in the juvenile fish was undamaged. However, the juvenile fish exhibited a slight tendency, only during the first test, to follow less well the movement of the vertical stripes than did the controls. Because these changes were below the significance level of the method, it can only be concluded that the otolithic system was essentially intact and operational in the recovered fish.

Parabolic flight tests.—Several parabolic flight tests were made with juvenile normal *Fundulus* fish, control fish of different ages, and flight juvenile fish. The tests showed that during the 25-second weightless periods, most *Fundulus* fish display the looping behavior at one time or another, as described earlier by von Baumgarten et al. (ref. 18-5).

Looping in weightlessness usually occurs when the fish start to swim forward spontaneously. Fish that are at rest or are trapped in corners or folds of the aquarium or bag often do not loop. The rate of looping typically increases during one looping episode. The direction of looping is mostly forward but it can be sideways in some fish. When the aircraft increases velocity during the parabola and the air bubbles in the fish tank indicate the presence of a longitudinal (X-axis) gravitoinertial vector, the fish turn their bellies into the direction of the gravitational pull and loop sideways.

Some fish occasionally roll around the longitudinal axis instead of looping. The roll direction of these "twisters" can change from clockwise to counterclockwise; however, one direction was always prevalent for any one fish. The opposite direction of roll usually started when the original roll was suddenly stopped by an obstacle (folds or corners of fish container). A possible explanation for the alternating direction of roll would be that in weightlessness the first roll movement starts because of a weight difference (imbalance) between the two utricular otoliths. The compensating impulses, which allow the proper equilibrium on the ground, would cause an imbalance in weightlessness. A sudden stop of such a roll movement would cause postrotational effects in the semicircular system and consequently would be followed by temporary counterrotation of the fish.

The experimental juvenile fish tested in parabolic aircraft flight 4 days after recovery looped as much as the control fish. It can be concluded from this that after 4 days on the ground, there were no "savings" preserved in these fish that would allow them to swim more normally in weightless conditions.

Light-orientation tests.—The results of light-orientation tests on the juvenile fish are presented in table 18-III as the group means of the roll angles for the light orientations. The C-I group was omit-

TABLE 18-III.—Group Responses to Light-Orientation Tests of Juvenile Fundulus Heteroclitus

Group	N[a]	Roll angle about body axis, deg				
		Trial 1	Trial 2	Trial 3	Trial 4	Mean
Left						
A	7	34.3	27.9	36.4	−10.7	22.0 ± 19.2
C-II	12	12.1	34.6	22.5	30.4	24.9 ± 8.6
C-III	6	23.3	2.5	35.8	35.8	24.4 ± 13.6
Right						
A	7	−8.6	7.1	−21.4	−20.7	−10.9 ± 12.6
C-II	12	−26.2	−23.8	−7.9	−18.3	−19.1 ± 7.1
C-III	6	−3.3	−39.2	−16.7	−29.2	−22.1 ± 13.5

[a]Number of samples.

ted because only four juveniles survived and two died midway through the test period. The roll angles were highly variable for all groups, and the mean values for each group for four trials were similar. There were no significant differences. The ASTP flight juveniles were slightly more variable, particularly for 90° light orientations on the right side of the fish, but this deviation is mentioned only for the sake of data completeness.

The light orienting of the *Fundulus heteroclitus* appears to be much more stable in adults than in juveniles.[1] Several competing responses in the juvenile *Fundulus* apparently increase the difficulty of obtaining a reliable measure of the roll orientation. It is possible to place an adult in a clear plastic cylinder and position its body perpendicular to the light rays for 30 minutes, thereby enabling observation of a slow roll to a final angle toward the light. However, the juvenile will frequently bend itself into a U-shape or become wedged upside down in the same type of tube.

On mission day 9, an attempt was made to measure the light-orienting responses of the groups of juveniles in each of the five compartments. The lights were turned off in the spacecraft, and the camera photolight was held at a 45° angle from the camera line of sight to the fish packages. The crewmembers reported that the fish did not orient themselves with respect to light direction but seemed to be alining their bodies with respect to the "bottom" of the fish packages adjacent to the spacecraft wall. It is difficult to believe that light direction was not the major influence for orientation while the fish were in a weightless environment, but it may be possible that a contrast gradient due to the cards in the "bottom" of the package or some type of tactile reference was the major influence. The crew reported that this light-orientation experiment was photographed but this cannot be confirmed from present techniques for viewing the flight films.

Otolith structure.—Because of the negligible effects of flight on orientation capability of either juveniles or hatchlings, it was considered unlikely that otolith degeneration would have occurred to a measurable extent. However, because of the availability of the material and the belief that some information on the otolith form could be useful in future studies, limited examinations were conducted.

Because the standard staging tables identify fish only during the embryonic period (while contained within the egg membranes), fish for baseline experiments were selected by size rather than developmental stage. The otoliths of *Fundulus* hatchlings that were less than 10 mm in body length; swimming fry of 20, 30, and 40 mm; juvenile fish of 50, 60, and 70 mm; and mature adults of 80, 90, and 100 mm were dissected from

[1]Hoffman, unpublished observations.

living animals and fixed immediately in cold 2.5 percent glutaraldehyde fixative in standard incubation medium for 24 hours. Following a wash in distilled water, they were postfixed for 1 hour in 1 percent osmic acid. The washed and dried specimens were coated with evaporated gold-palladium and examined at low to moderate magnification in a scanning electron microscope.

Following the return of the flight fish, the otoliths were compared by the established procedure with otoliths from control animals maintained on Earth. Light micrographs of vestibular systems dissected from the right sides of flight and control juveniles are shown in figure 18-3.

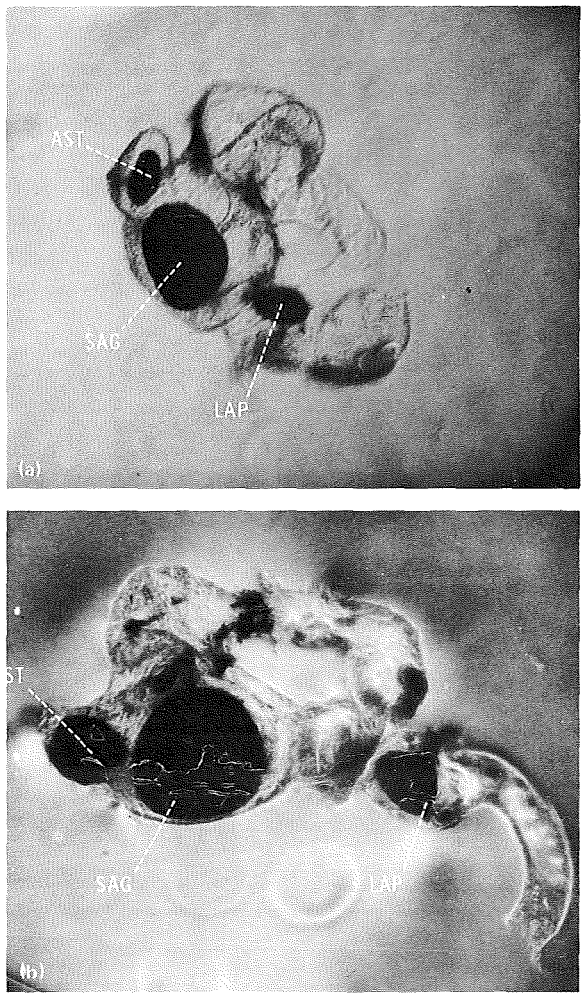


FIGURE 18-3.—Light micrographs of dissected vestibular tissues from the right sides of fish. LAP, lapillus; SAG, sagitta; AST, asteriscus (52×). (a) Control fish. (b) Flight fish.

Both fish were 12 mm long. The large (sagittae) stones are shown as they appear at 200× magnification under a scanning electron microscope (figs. 18-4(a) and 18-4(b)). The medial surfaces shown are characteristically grooved and sculptured; the lateral surfaces (not shown) are smooth with concentric "growth rings." In larger

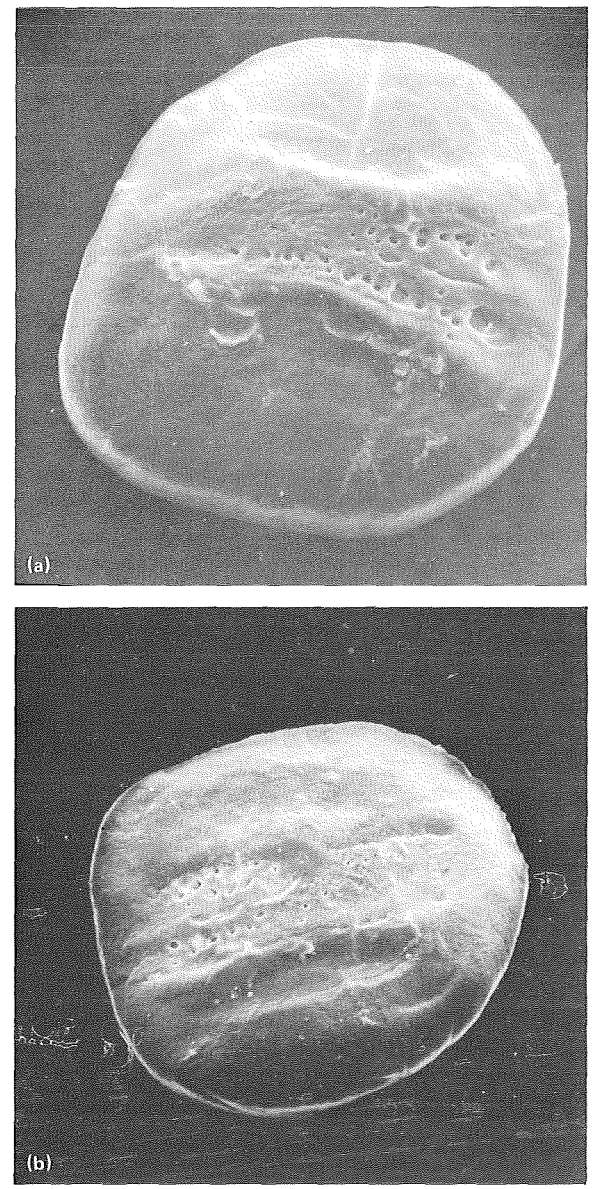


FIGURE 18-4.—Scanning electron micrographs of sagittae. (a) Medial view of left sagitta of flight juvenile (200×). (b) Medial view of right sagitta of controlled juvenile (200×). (c) Medial groove of a 30-mm-long juvenile (1240×).

FIGURE 18-4.—Concluded.

fish, "crystalline" projections appear in the groove as shown in the scanning micrograph of a stone from a 30-mm-long fish examined during baseline studies (fig. 18-4(c)). None of the scanning electron microscopic examinations revealed recognizable differences in size, shape, or surface structure between flight and control juveniles.

Embryo Development

Development rate.—It was recognized at the outset that lack of synchrony in development could present problems in the interpretation of results. Although fertilization of all embryos in a given age group occurred within the space of a few minutes, variability in development rate was such that two to three distinguishable developmental stages were represented in this group at any given time.[2] Thus, it was possible to know only as an average the developmental stage at which each age group was exposed. Theoretically, this point could have been quite important; however, operational constraints prevented the selection and flight exposure of specific developmental stages; and, for the purposes of this particular experiment, the practical significance seems to have been relatively small.

Hatching, similar to development, is a variable affair. A population of embryos that theoretically has developed more or less uniformly to the point of hatching at between 9 and 15 days will, if left undisturbed, emerge a few at a time over a period of several weeks. Under such circumstances, the uniform sampling and testing of experimental animals would have been a tedious and complicated operation at best. It was found, however, during the course of preflight baseline testing, that if fully developed but sporadically emerging embryos were subjected to a short period (5 to 20 minutes) of vigorous agitation, all individuals apparently physiologically ready to emerge would do so within a period of approximately 20 minutes following cessation of the agitation. Conversely, embryos grown under conditions of continuous agitation would commence wholesale emergence within a short time after cessation of agitation.[3] The circumstantial evidence suggests that one strong stimulus to emergence is a change in the pattern of vestibular stimulation.

By employing the periodic shaking technique, the majority of an age group could be induced to emerge over a period of a few days by a regimen consisting of 20 minutes of vigorous agitation of the culture vessel every 24 hours. (Typically, this was accomplished in 100-mm petri plates on a rotary shaker at 100 excursions per minute.) Thus, the process of hatching could be compressed into a minimum time and, in addition, a means was available for positively assessing the end point of embryonic development in terms of ability to emerge.

A real count of hatching was rendered impossible by the sampling of unhatched eggs for microscopic study, but a relatively close approximation was obtained by applying the formula

$$T_n = T_{n-1} + O_n + \frac{O_n}{R_n + O_n}\left(F_n - S_n\right) \qquad (18\text{-}1)$$

[2]Boyd, unpublished observations.

[3]Scheld and Boyd, unpublished observations.

where $T_{1,2,3,...n}$ is the theoretical cumulative hatch for days 1,2,3,...n; $O_{1,2,3,...n}$ is the observed hatch on days 1,2,3,...n; $R_{1,2,3,...n}$ is the total unhatched individuals remaining after shaking on day 1,2,3,-...n; $F_{1,2,3,...n}$ is the sum of unhatched eggs fixed up to the day selected; and S is the sum of the fixed individuals (eggs) that had been calculated as theoretically hatched up to the day in question.

These data were analyzed by methods described by Baky (ref. 18-11) for analysis of binomial data. Comparisons were made of ultimate hatch and of 75 percent hatch time as an index of hatching rate. The results of these comparisons are summarized in tables 18-IV and 18-V.

There is some small possibility that the atmospheric environment of the spacecraft may have been more favorable for development, as indicated by slightly higher hatch in the C-I and C-III treatments that attempted to duplicate spacecraft environments. In general, however, the final hatch of the flight group was significantly different from the pooled controls at the 5-percent level for at least the youngest age groups (table 18-IV). Pooled 66-hour controls missed significance by only a very small margin. If ultimate development can be considered an index of health, and anomaly rates are not different, then it seems reasonable to conclude that flight has had some beneficial effect on health of the organisms. And if the assumption is made that onset of ability to hatch is an index of overall development rate, then it must be concluded that there was a definite

TABLE 18-IV.—Percent Final Hatch for Flight, C-I, C-II, and C-III Groups Over the Five Development Stages Exposed to Zero-g Conditions and Allowed to Continue Development for the Duration of the 9-Day Mission

Development stage, hr	*Hatchability, percent*			
	Flight	*C-I*	*C-II*	*C-III*
32	[a]100	94	96	[b]94
66	[a]97	93	93	[a]96
128	[a]99	[a]100	88	[a,b]98
216	[a]100	[a]98	92	[a,b]98
336	100	98	98	98

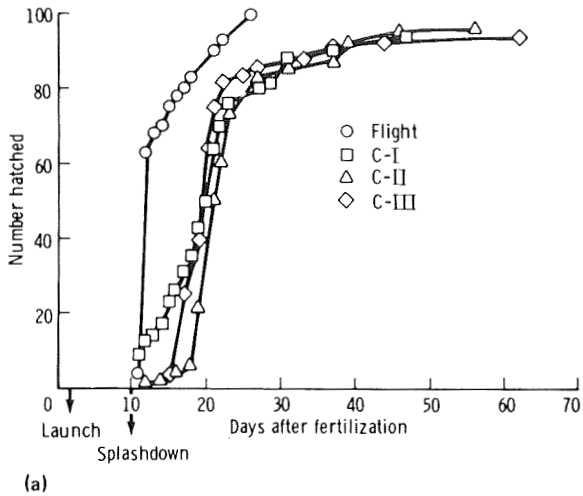
[a]Significant at the 5-percent level.
[b]Pooled controls that were significantly different.

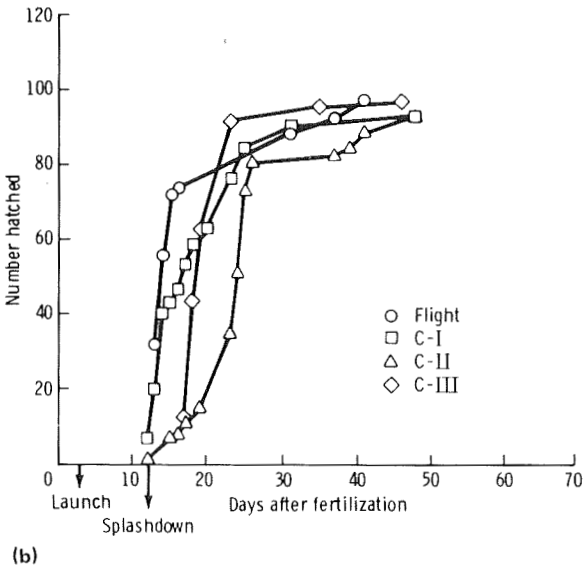
TABLE 18-V.—Time to Achievement of 75 Percent Hatch for Flight, C-I, C-II, and C-III Groups Over the Five Development Stages Exposed To Zero-g Conditions and Allowed to Continue Development for the Duration of the 9-Day Mission.

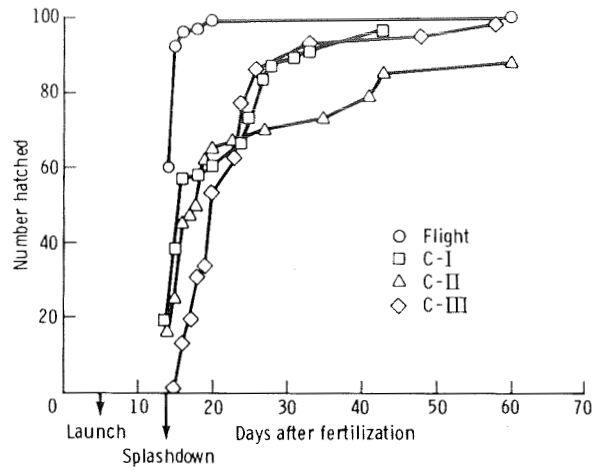
Development stage, hr	*Time to 75 percent hatch, days*			
	Flight	*C-I*	*C-II*	*C-III*
32	[a]15	23	26	21
66	[a]15	23	26	21
128	[a]15	25	27	24
216	18	19	19	22
336	23	23	24	25

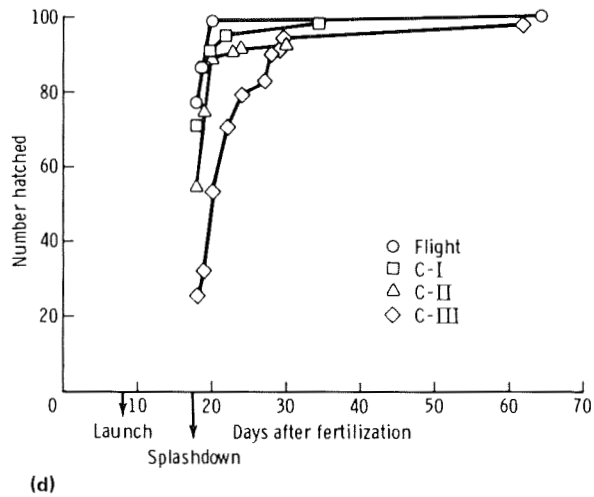
[a]Significant at the 1-percent level.

trend toward a higher rate of development in flight-exposed eggs. The trend is quite apparent in plotted cumulative hatching curves (fig. 18-5) and is statistically significant at the 1-percent level when 75 percent hatching times are compared.









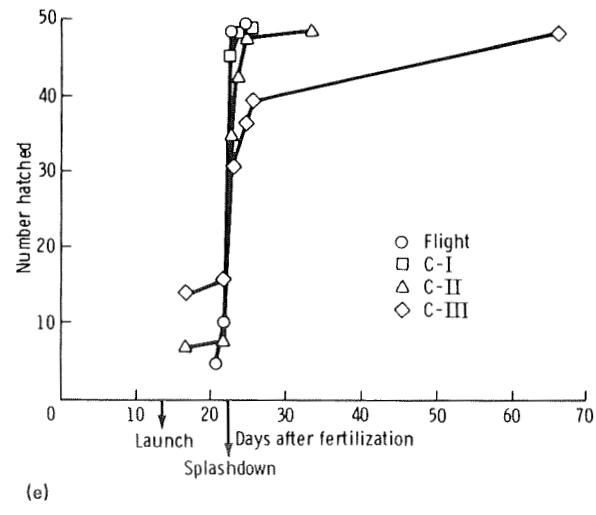


FIGURE 18-5.—Cumulative hatching curves of eggs from flight and control treatment packages. (a) 32-hour eggs. (b) 66-hour eggs. (c) 128-hour eggs. (d) 216-hour eggs. (e) 336-hour eggs.

FIGURE 18-6.—Photographs of flight and control animals taken mission day 8. (a) Flight juveniles that were preconditioned in a vertically striped environment are in compartment 2, those preconditioned in a horizontally striped environment are in compartment 3. (b) Flight 66-hour eggs in compartment 2 and 128-hour eggs in compartment 3. (c) Flight 216-hour eggs in compartment 4 and 336-hour eggs in compartment 5. (d) Control III 216-hour eggs in compartment 4. (e) Control III 336-hour eggs in compartment 5.

Similar observations of increased rate or efficiency of metabolic activity may have been made in Biosatellite II experiments; for example, the increased density of *Salmonella* growth (Experiment P-1135) (ref. 18-12) or increased *Tradescantia* flower production (Experiment P-1123) (ref. 18-13). Factors related to the promotion of more favorable conditions for dissipation of toxic metabolites or more efficient utilization of oxygen by the very even distribution of eggs within the available fluid volume (fig. 18-6) may have been solely responsible. It seems possible, however,

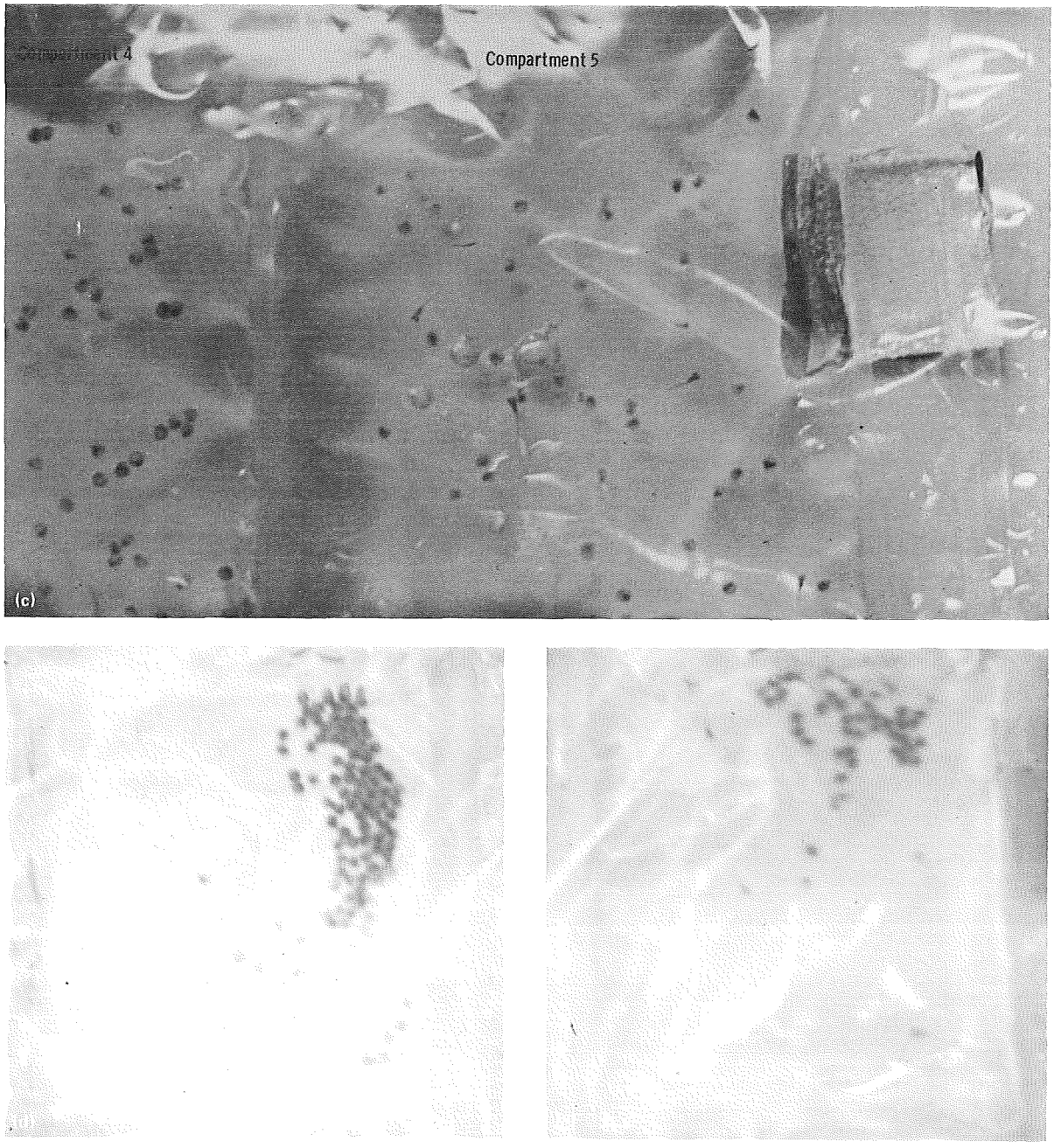


FIGURE 18-6.—Concluded.

FIGURE 18-7.—Cross section of the head region of flight and control hatchlings at the level of the utricle (ASC, anterior semicircular canal; U, utricle; LAP, lapillus (utricular otolith); HC, hair cell, OM, otolithic membrane; arrow, metachromatic dense bodies). (a) Flight 128-hour sample at 20× magnification. (b) Flight 128-hour sample at 200× magnification. (c) Flight 128-hour sample at 200× magnification. (d) Control I 128-hour sample at 200× magnification.

FIGURE 18-7.—Concluded.

that absence of the necessity to expend energy to maintain structural integrity against gravitational force could also impart a small but additively significant advantage in growth rate. Although a profound biological significance cannot be attached to this observation, it is worth noting as at least a possible indirect effect of zero-g conditions, which could have significance in the future for applied aspects of space flight.

Microscopic examinations.—Initial histological and ultrastructural analyses of the four oldest age categories (66 hour through 336 hour) have been completed, and no significant differences between flight and ground control treatments were detected in the embryological development of the central nervous system, the peripheral vestibular apparatus, the eye, or the cardiovascular system. In all of these groups, the peripheral vestibular system developed fully with complete expression of the maculae of the sacculus and utriculus (figs. 18-7 and 18-8). The statoliths of the sacculus (*lapillus*) and utriculus (*sagitta*) followed normal sequence development, and the lagenal statolith (*asteriscus*) initiated development between posthatching days 4 and 6 in all groups (figs. 18-9(a) and 18-9(b)). Except for a decrease in the extent and content of the vitreous cavity, ocular development was judged to be normal (figs. 18-9(c) and 18-9(d)). Difficulties in maintaining the proper components within the fixative solution during the long preflight storage interval apparently contributed to osmotic collapse of the

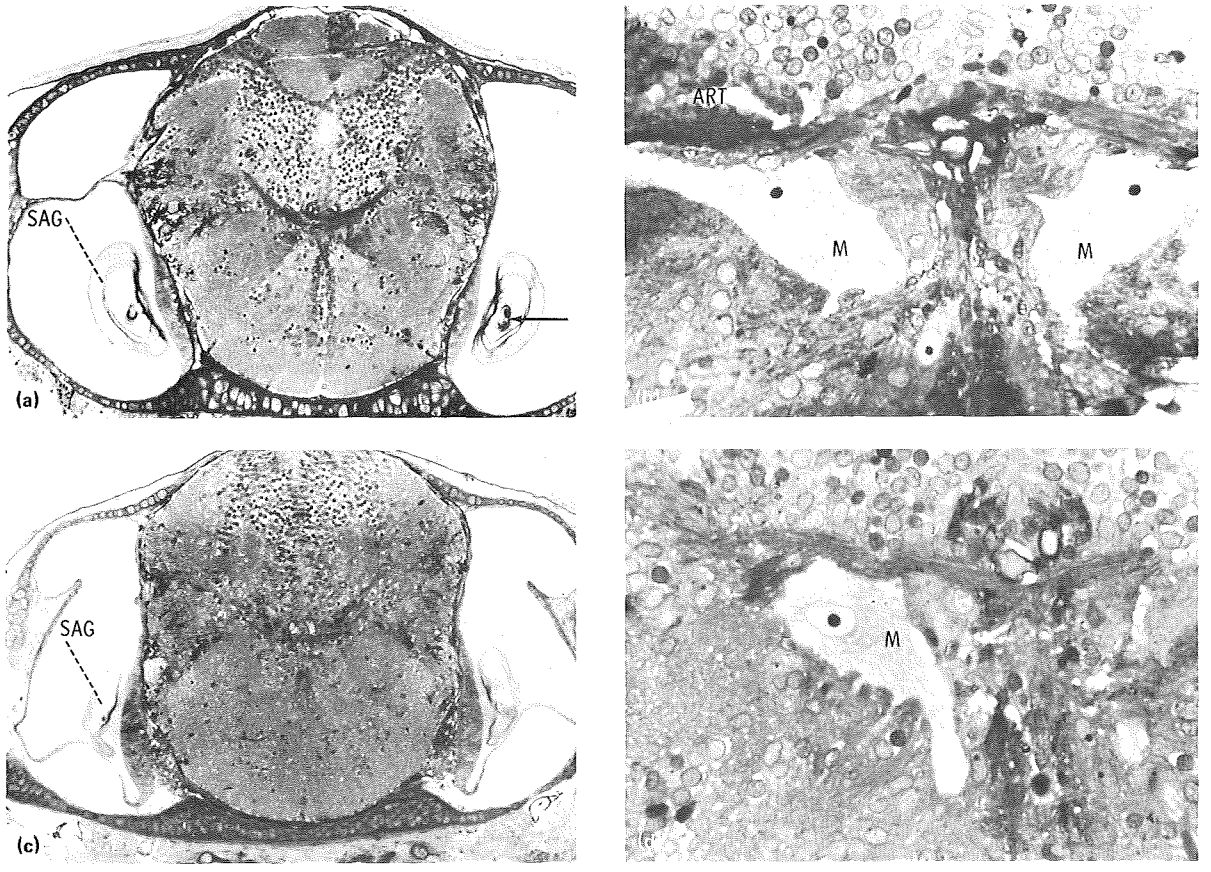


FIGURE 18-8.—Cross section of the head region of flight and control hatchlings at the level of the sacculus (SAG, sagitta (saccular otolith); M, Mauthner cell; ART, process artifact; arrow, metachromatic dense body). (a) Flight 128-hour sample at 23.8× magnification. (b) Flight 128-hour sample at 90× magnification. (c) Control 128-hour sample at 23.8× magnification. (d) Control 128-hour sample at 90× magnification.

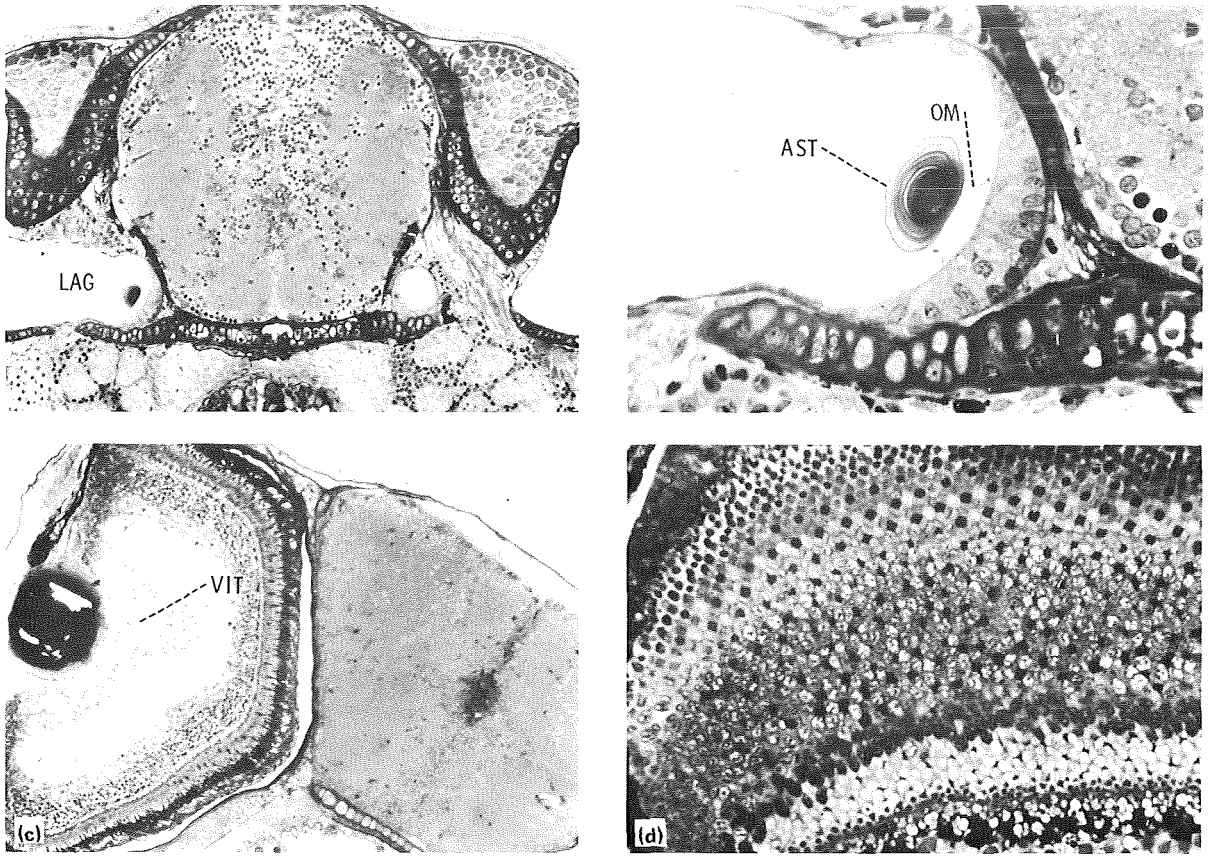


FIGURE 18-9.—Cross sections of the head regions of flight hatchlings at the level of lagena and the eye (LAG, lagena; AST, asteriscus (lagenar otolith); VIT, vitreous body; OM, otolithic membrane; ART, process artifact). (a) Flight 128-hour sample showing the lagena at 24.4× magnification. (b) Flight 128-hour sample showing the lagena at 92× magnification. (c) Flight 128-hour sample showing the eye at 24.4× magnification. (d) Flight 128-hour sample showing the retina at 92× magnification.

eyes during fixation and to pooling of plasma proteins in both experimental and control groups of animals. Ultrastructural analyses of the developing statoliths and sensory maculae have revealed no differences between matched groups of animals (figs. 18-10(a) and 18-10(b)). Both sustentacular and hair cells followed normal developmental parameters. Central projections and vestibular ganglia contained normal cellular complement and projection configuration. Cartilage development and calcification was identical in flight and control categories (fig. 18-10(c)).

Preliminary analysis of the specimens treated with tritiated thymidine at selected post-splashdown periods has also failed to reveal any significant alterations in the patterns of cellular proliferation within the vestibular, ocular, or central nervous systems of the oldest stages flown. Cells of the ependyma and cortical neuroepithelium, the *planum semilunatum* of the maculae, and the *ora serrata* of the retina were heavily and consistently labeled in all groups. Further analysis of these animals is now underway to ensure statistical confidence in the morphological observations.

The youngest age category flown, 32 hours after fertilization, was at the mid-to-late gastrula stage at the time of lift-off and orbital insertion, and would be expected to reveal the most significant effects of such an experience. Analysis of these animals is still in progress.

Several factors must be pointed out with

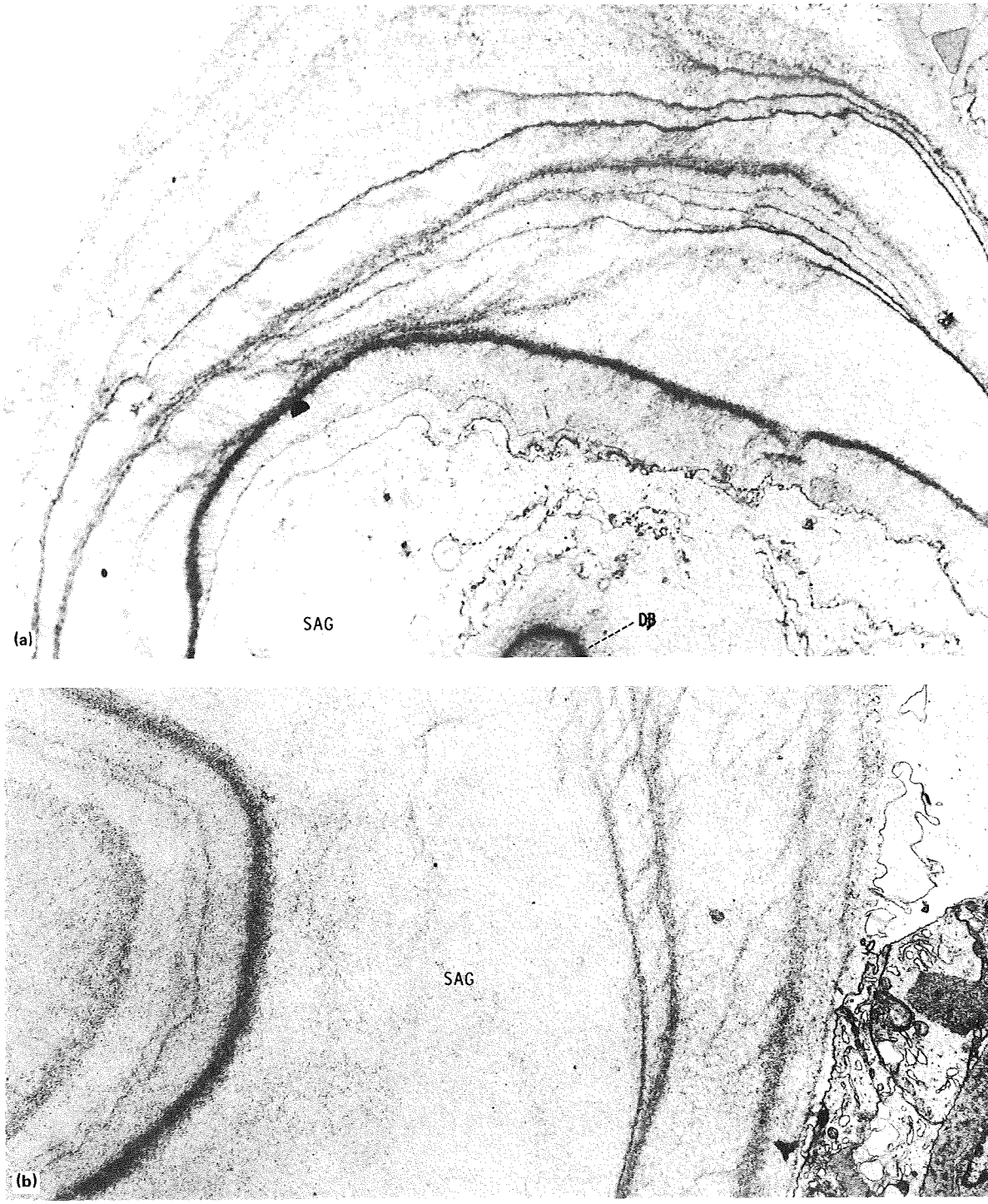


FIGURE 18-10.—Electron micrographs of calcified areas in the vestibular region of flight and control hatchlings (CH, chondrocyte; FIB, fibrocyte; CAL CART, calcified cartilage; DB, metachromatic dense body; SAG, sagitta (saccular otolith)). (a) Saccular otolith of flight 128-hour sample at 6000× magnification. (b) Saccular otolith of control 128-hour sample at 6000× magnification. (c) Calcified cartilage of flight 128-hour sample at 32 000× magnification.

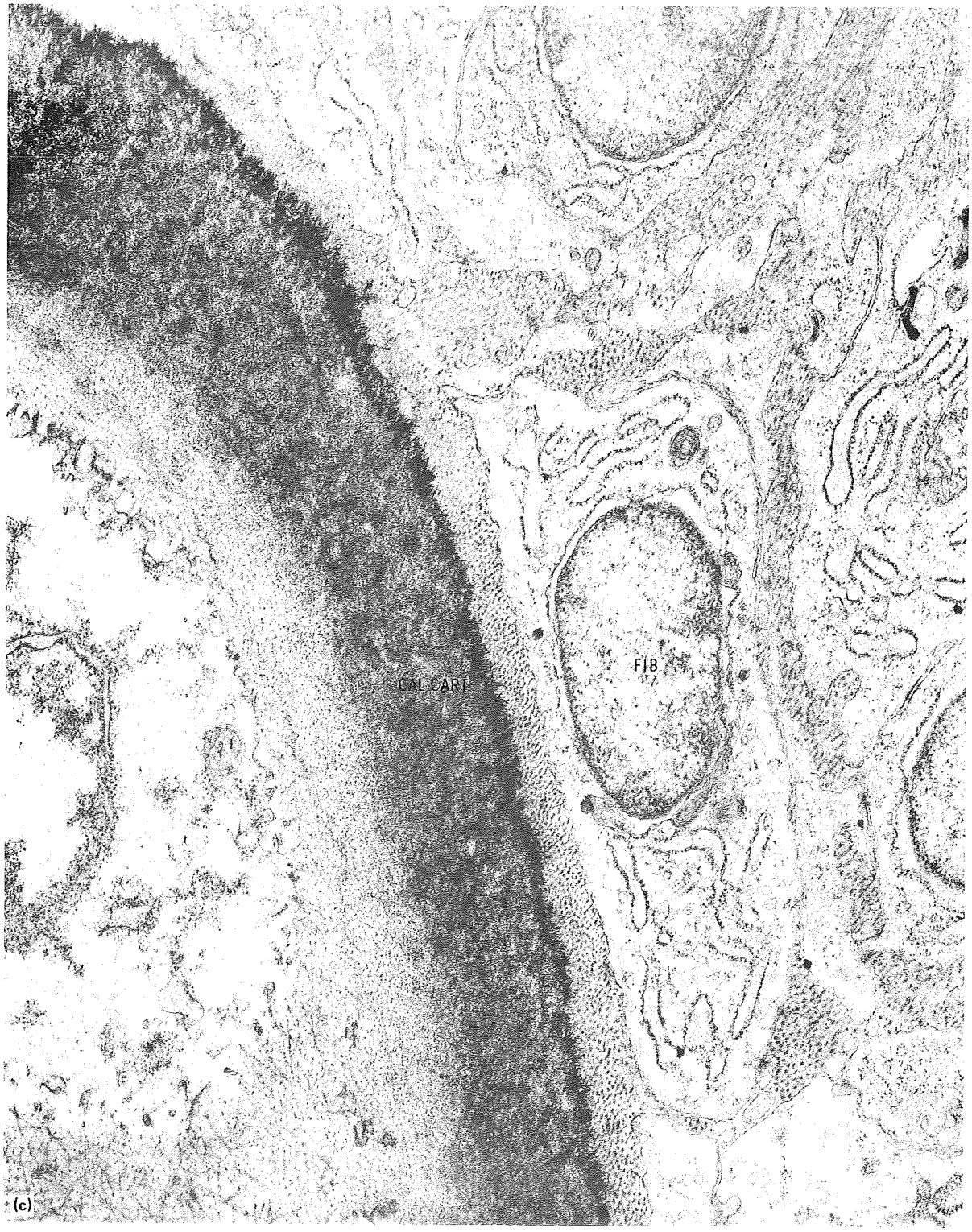


FIGURE 18-10.—Concluded.

respect to the normal development of the *Fundulus heteroclitus* embryos in this experiment. *Fundulus* eggs develop freely within a natural aquatic environment and as such are not dependent on a maternal-fetal interaction. Such a system would actually be facilitated by the conditions found during space flight in which the eggs are randomly dispersed throughout the aquarium and the diffusion and distribution of metabolites is assured. The necessity of packaging and transporting to launch site precluded the analysis of earlier, potentially more susceptible, stages of development. The earliest stages achievable were already mid-to-late gastrulae at the time of insertion, well past the prime sequence of most critical embryonic interactions with respect to vestibular initiation, retinal and lens inception, and central nervous system inductions. Finally, the duration of the mission (9.5 days) is a minimal-duration period of sensory deafferentation.

The foregoing factors should not detract, however, from the primary critical significance of the normal development of these embryos during conditions of space flight. They should, however, suggest the continuing need for a critical analysis of longer term earlier stage embryos as well as the need to extend these studies to a model system entailing maternal-fetal interactions.

Vestibular behavior in flight.—Theoretically, three out of the five age groups should have been capable of hatching in flight at some time during the 9-day mission. In fact, only approximately 20 percent of the oldest group (the 336-hour eggs) hatched during flight. The eggs of this group were fully developed but unhatched at the time of launch. The delayed hatching observed on Skylab 3 was thus confirmed. The probable reasons for delay have already been discussed in the preceding subsection. The observed reaction supports the theory that vestibular stimulation is a cue for emergence.

No looping behavior was observed in hatchlings on Skylab 3. This was originally interpreted as an indication of either the absence of a functional vestibular system or of complete habituation of the hatchlings. The 336-hour hatchlings that did emerge during flight exhibited occasional looping behavior, as reported by the crew. The fact that no looping of these animals was observed in flight films would suggest that habituation of the hatchling fish might have been somewhat better than that of juveniles. Because no hatching occurred in any of the younger groups, it is still not possible to confirm or reject the Skylab observations. The 128-hour group would have been most similar to the Skylab animals.

Recovery.—Although little hatching occurred during flight, the stimulation of handling subsequent to splashdown, the agitation caused by movement of the packages during initial photography, and the standard stimulating shake were enough to cause wholesale emergence of all but the youngest group (fig. 18-5).

The control treatment groups, which had been maintained under static conditions, exhibited similar but less striking emergence patterns. As noted in the discussion of hatching rates, hatchlings exhibited a strong diving response. Testing on the rotating striped drum indicated normal reaction, with the possible exception of the 128-hour postshake hatchlings, which exhibited a slightly increased tendency to follow the stripes in the initial test only, suggesting a possible temporary hyposensitivity of the vestibular system.

Flight hatchlings exposed to short periods of weightlessness during parabolic flights at 5 days past recovery also exhibited looping behavior that was indistinguishable from that of the controls. Thus, whatever habituation might have been observed during exposure in flight was lost soon after return to a one-g environment.

Postflight vestibular testing.—At approximately 6 months of age, the hatchlings from the flight treatment group were subjected to a geotaxis test. The test consisted of placing each fish in a filled 500-ml graduated cylinder and measuring the amount of time spent in the upper half during a 2-minute interval. The test was administered three times to each fish in the 32-hour, 66-hour, and 128-hour groups after preconditioning under two separate light-dark cycles (12:12 and 16:8). The 32-hour flight fish were found to spend, on the average, 70 percent more time in the upper half of the graduated cylinder than did the control fish ($p < 0.05$). There were no significant differences for the other two groups (table 18-VI(a)). For all three flight treatment groups there was a highly correlated relation ($r = 0.85$) between the number

TABLE 18-VI.—Analysis of Geotactic Response in 6- to 8-Month Hatchlings From Flight and Control Treatment Groups for Four Variables[a]

(a) Comparison of treatment effects

Development stage, hr	L(1)		L		W		G	
	Apollo	Control	Apollo	Control	Apollo	Control	Apollo	Control
32	34.33	31.91	28.28	26.91	419	345	[b,c]20.07	11.49
66	[c]36.54	[c]36.54	[c]30.00	[c]30.38	[c]515	[c]531	8.82	10.11
128	30.75	32.63	25.58	27.03	312	385	9.18	9.18

(b) Correlation analysis including a fifth variable n[d]

Variable	Apollo					Control				
	G	n	L(1)	L	W	G	n	L(1)	L	W
G	1.00	[e,f]0.85	−0.53	−0.53	−0.56	1.00	[f]0.12	−0.34	−0.32	−0.38
n	[e,f].85	1.00	[e]−.79	[e]−.79	[e]−.83	.12	1.00	[e]−.88	[e]−.86	−.85
L(1)	−.53	[e]−.79	1.00	[e]1.00	[e].99	−.34	[e]−.88	1.00	[e].99	[e].97
L	−.53	[e]−.79	[e]1.00	1.00	[e].99	−.32	[e]−.86	[e].99	1.00	[e].94
W	−.56	[e]−.83	[e].99	[e].99	1.00	−.38	[e]−.85	[e].97	[e].94	1.00

[a]G, geotaxis; L(1), length; L, length without tail; W, weight.
[b]Significant; different from control.
[c]Significant; different from other stages.
[d]Number of fish per tank.
[e]Significant at the 5-percent level.
[f]Significant difference between flight and control fish.

of fish in a home tank and the geotaxis parameter under study (table 18-VI(b)). This correlation did not occur for control fish and cannot be explained in terms of any size differences.

The diving response is very strong in most *Fundulus heteroclitus* and, therefore, was considered to be a measure of whether or not the innate vestibular programing was modified by an exposure to null-gravity conditions during embryonic development. The finding of a behavioral difference in the 32-hour group was not unexpected, because behavioral testing is a sensitive measure of subtle effects of environmental influences. The 32-hour and 66-hour groups were considered more likely to be affected in a minor way than other developmental stages, because the vestibular apparatus was not completely formed at the time normal gravity influence was removed. The correlation between population size and orientation behavior was, however, unexpected. Considering this finding and the observations that the geotaxis parameters for experimental fish, but not control fish, tended to vary with the photoperiod (although not significantly), there is an indication that the experimental fish are more sensitive to environmental factors (at least for the case of crowding and photoperiod). These findings are minor, but further investigation is needed to ensure that exposure to weightlessness during critical periods of development does not produce any profound effects.

ACKNOWLEDGMENTS

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